The direct electrochemistry of cytochrome P450. What are we actually measuring?

Michael Honeychurch

m.honeychurch@uq.edu.au

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Abstract

The direct electrochemistry of cytochrome P450 has been reported in published articles over the last ten years but has never been conclusively demonstrated because generally none, at best few, but never all, of the necessary control experiments required to claim success in this area have ever been carried out and reported in published work. The objective of this article is to describe why many additional experiments are necessary and to recommend a systematic set of control experiments that are required before the statement that “direct electrochemistry of cytochrome P450 has been achieved” can have some validity.

Introduction

The cytochrome P450 (P450) family of monooxygenase enzymes has been much studied over the last 30 years [1-3]. The enzymes catalyse the hydroxylation of their substrates via an oxygen atom insertion.

A typical representation of the P450 catalytic cycle is shown in Figure 1.

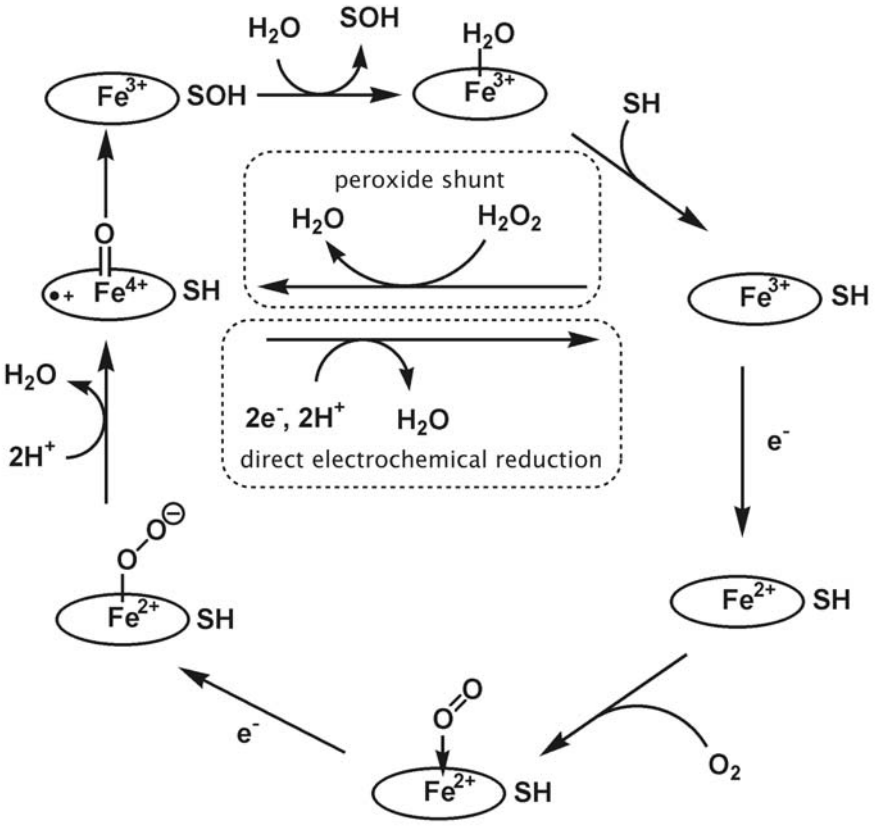

Figure 1. Proposed cytochrome P450 catalytic cycle including the "peroxide shunt" mechanism in which the high valent oxyferryl (Fe^{4+}=O) is formed from the reaction of hydrogen peroxide and Fe^{3+}. Direct electrochemical reduction of (Fe^{4+}=O) to Fe^{3+}, a potential "short circuit" mechanism, is also shown. SH is the substrate.

The direct electrochemistry of P450 has been attempted often and published voltammetric results following the immobilisation of P450 on electrode surfaces have recently been surveyed [4]. The general conclusions that are formed and stated in published articles is that a voltammogram showing a pair (reduction and

subsequent oxidation or vica versa) of peaks in an approximate potential region where the P450 redox potential has been measured potentiometrically or spectroscopically indicates that the direct electrochemistry of cytochrome P450 has been achieved. In reality this result should merely be the starting point of the investigation.

An interesting aspect of P450 chemistry is that under certain conditions P450 can irreversibly change to an enzymatically inactive – toward the natural substrate – form known as cytochrome P420. It is so named because of the sharp Soret peak of the CO bound reduced form at 420 nm – whereas P450 has a Soret peak of the CO bound reduced form at 450 nm. The P450 to P420 transformation can occur as a result of many things such as exposure to organic solvent and variations in pH. P420 can also be prepared deliberately using well established methods [5] and used as a control in P450 experiments, though this has never been reported in the literature.

To date, in the published work, no systematic control experiments have been carried out in order to eliminate the possibility that the observed electrochemical response is due to such things as cytochrome P420 or displaced heme, or that measured catalysis is due to hydrogen peroxide formed following the electrochemical reduction of dioxygen – the so-called peroxide shunt mechanism. In the authors opinion the direct electrochemistry of cytochrome P450 has never been conclusively demonstrated because generally none, at best few, but never all, of the necessary control experiments required to claim success in this area have ever been carried out and reported in published work. The objective of this article is to describe why many additional experiments are necessary and to recommend a systematic set of control experiments that are required before the statement that “direct electrochemistry of cytochrome P450 has been achieved” can have some validity.

The focus of this review is limited to direct non-mediated electrochemistry. We begin in the next section by discussing the thermodynamics and kinetics of P450. This is followed by a discussion of direct electrochemical experiments and observations of the “non-turnover” electrochemical response, i.e. the electrochemical response in the absence of substrate. Since P450 catalysis requires

the presense of dioxygen the inflence of dioxygen on the P450 electrochemical response is discussed. Finally we consider how one might determine if electrochemically driven catalysis is taking place.

P450 kinetics and thermodynamics

Bacterial cytochrome $P450_{cam}$ ($P450_{cam}$) from *Pseudomonas putida* has been one of the most studied of the P450 enzymes. $P450_{cam}$ receives two electrons from its redox partner, putidaredoxin, in two separate steps during the catalytic cycle as shown in Figure 1. The two separate electron transfer steps have been shown to be the slowest steps in the $P450_{cam}$ catalytic cycle. The rate of the first electron transfer from putidaredoxin to $P450_{cam}$ is considerably slower in the absence of its substrate, camphor, compared to electron transfer with the camphor-bound enzyme.

When substrates bind to P450s a large positive shift in what is referred to in the electrochemical literature as the formal potential, $E^{0'}$, and in the biological literature as the midpoint potential, E_m, is reported to occur. The $E^{0'}$ and E_m potentials are the redox potentials *in the prevailing medium*. In order to make conclusions about the thermodynamics of a reaction one needs to know $\Delta G^{0'}$[†], the free energy of the reaction in the prevailing medium. The value of $\Delta G^{0'}$ can be obtained from concentration measurements in the prevailing medium or from redox potential measurements (i.e. $E^{0'}$, E_m).

A measurement of the redox potential in an anaerobic medium may tell us that a reaction is thermodynamically unfavourable in that medium but when it is known that dioxygen binds strongly and rapidly to one of the redox states the anaerobic measurement tells us nothing about the reaction in an aerobic environment. In fact the reporting and subsequent analysis of anaerobic redox potentials has simply served to produce erroneous conclusions that have been passed down in the

[†] The prime is used here to indicate that it is not a standard state.

literature.

The anaerobic potential shift upon substrate binding has been offered as a reason for the differences in the respective NADPH turnover rates of substrate bound and substrate free P450. It is been suggested that the reaction of P450s with their redox partner become thermodynamically favourable following the redox potential shift that occurs following anaerobic substrate binding. This has been (mis)interpreted as a kind of natural thermodynamic switch. The P450 catalysis requires not only the binding of the substrate but also the binding of a "co-substrate", dioxygen, to reduced iron and this dioxygen binding can occur regardless of whether substrate is present. Measurements of the redox potentials of P450s that are reported in the literature are made anaearobically because unfortunately the experimental methods used to determine the redox potential typically requires the exclusion of dioxygen. The exclusion of dioxygen from the redox potential measurement produces a measurement in an experimental medium that is unrelated to the prevailing medium during real life enzyme catalysis (i.e. an aerobic medium). Just as binding of carbon monoxide to the ferrous P450 causes a positive shift in the redox potential, the binding of dioxygen to ferrous P450 also must cause a positive shift in the potential. The positive potential shift is driven by the greater affinity of dioxygen (and CO) for the reduced form of the enzyme relative to the affinity for the oxidised form. If a ligand has a greater affinity for the product of a reaction the equilibrium for that reaction is shifted to the right. This should be self evident given Le Chataliers principle. Thus when we consider the Fe^{3+} to Fe^{2+} redox reaction occuring in P450s we see that the redox potential shifts positively in the presence of CO. If analogous measurements were possible in the presence of dioxygen (i.e. aerobic solutions) an analogous positive potential shift would be observed. It follows that the reduction potential of both substrate-bound and substrate-free P450 will be expected to increase (be more positive) in the presence of dioxygen. This increase in the reduction potential is likely to be sufficient to make electron transfer to substrate-free P450 thermodynamically favourable. These thermodynamic arguments suggest that electrons will be cycled in the absence of substrate, whether futile or not, and this is in fact what is observed.

The potential shift following substrate binding is often attributed as being due to the ferric spin-state shift that accompanies substrate binding. In the camphor hydroxylase $P450_{cam}$ the high spin/low spin distribution is dependent on the concentration of potassium ions. Therefore if the potential shift was spin state dependent it follows that it must be potassium ion dependent. On the other hand in many other P450s no change in spin states have been observed following substrate binding.

In the substrate-free form of P450 the ferric heme centre is six co-ordinate and predominantly low spin with a water molecule co-ordinated in the distal position. In the substrate-bound form the ferric heme centre loses the water molecule, becoming five co-ordinate and predominantly high spin. The heme iron in ferrous P450 is five co-ordinate and high spin in both its substrate-free and substrate-bound forms.

Given that the free energy changes, in electron Volts (eV), for the electron transfer reaction are only of the order of <100 meV the dominant term in the semi-classical Marcus equation for the rate, *k*, of electron transfer,

$$k \propto \exp\left(-\frac{\left(\lambda + \Delta G^{0'}\right)^2}{4\lambda k_B T}\right) \qquad (1)$$

is going to be the reorganisation energy, λ. (In this eqn (1) k_B is Boltzmans constant and T is temperature). In other words for the P450 electron transfer reaction the reorganisation energy will be significantly greater than the reaction free energy. When comparing the substrate bound and substrate free reactions the reorganisation energy for the substrate free reaction, in which the co-ordination changes from 6 to 5, is likely to be larger than in the substrate bound reaction, in which both oxidation states are 5 co-ordinate. Therefore qualitatively we would predict from the Marcus equation that the turnover rate in substrate free enzyme would be slower than substrate bound enzyme. This is what is observed experimentally and it is due not to a thermodynamic switch but to the difference in reorganisation energies in going from substrate bounde to substrate free. And what of the spin state change? Well we may expect some small differences in reorganisation energy due to the different

electron density “pattern” in different spin states but this difference should be relatively insignificant compared to the reorganisation energy change occuring from a change in co-ordination.

Direct electrochemical experiments with P450s

Electrochemical enzyme studies are typically performed to determine fundamental parameters, such as the redox potential of the enzyme, or to study the electron transfer between the enzyme and various electrodes, either directly or through the use of mediators [6]. In addition to fundamental studies, electrochemical studies of P450s are of great interest due to the possibility of developing applications such as biosensors for analyte detection and bioelectrochemical catalysts for product synthesis.

Attempts to electrochemically drive the P450 catalytic cycle seek to replace the natural redox partner with an electrode (Figure 2).

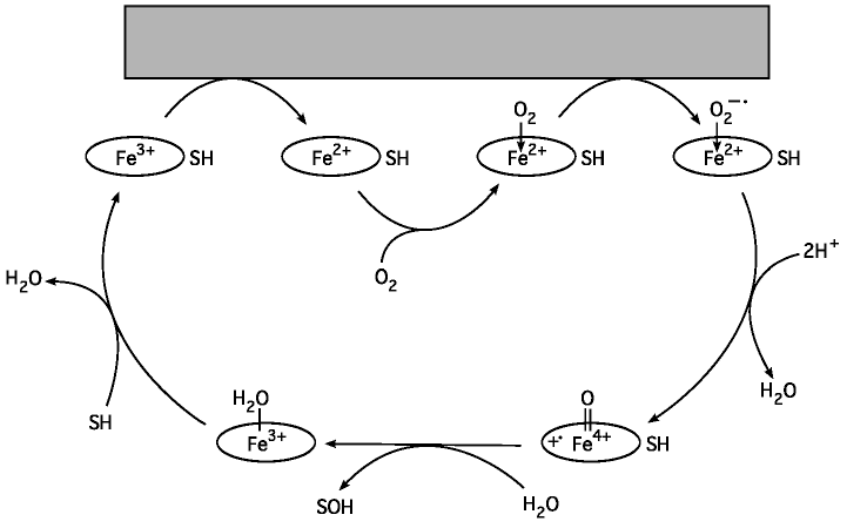

Figure 2. Proposed electrochemically driven P450 catalytic cycle. SH is the substrate.

The electrochemical behaviour of enzymes is more complicated that that of smaller molecules that have traditionally been the subject of studies by electrochemists. The most important complication is the possibility of conformation changes occuring

within the enzyme during the measurement process. How do we know that a non-turnover electrochemical response is due to the functioning enzyme and not some altered conformation of the enzyme in which its activity is reduced or eliminated altogether? Control experiments to test this possiblity are discussed below. Their is also a rather obvious potential problem that could cause the cycle to "short circuit." The proposed catalytic cycle shown in Figure 2 requires that the electrode potential be sufficiently negative to reduce Fe^{3+}. One would therefore think that in this environment the formation of a higher Fe oxidation state (Fe(IV)=O) to be unlikely or at best, since an overpotential (driving force) of ~ >1V is being applied, this state would be rapidly electrochemically reduced back to, firstly Fe^{3+}, and then Fe^{2+}, thereby short circuiting the catalytic mechanism. Surprisingly this prospect has remained uncommented upon. On one hand it may be argued that in Nature the chemical environment is sufficiently reducing to reduce Fe^{3+} yet this clearly does not inhibit the formation of the high valent oxyferryl intermediate. The difference with an electrochemically driven system is that the electric field being generated by the electrode is constant whereas the reduction by the natural redox partner (i.e. redoxin or flavoprotein) is not constant but on and off as the enzyme and redox partner bind and then tranfer an electron.

Assuming that the Fe^{4+}=O intermediate can form, the potential for short circuiting will only be significant if the rate of its direct electrochemical reduction is much greater than the rate of oxidation of the substrate. Before comparing these rates consider two studies using synthetic P450 analogues. Murray and co-workers successfully electrochemically mimicked the P450 catalytic cycle with a manganese porphyrin [7]. The electrochemical yield for olefin epoxidation was approximately 50% indicating that the rate of the oxygen insertion step was of the same order as the direct reduction of Mn^{5+}=O by the electrode. Khenkin and co-workers electrochemically mimicked the P450 catalytic cycle with an iron porphyrin [8]. The yield for the electrochemically driven oxidation of cyclohexane to cyclohexanol was 7%. The O-O bond cleavage in dioxygen was promoted by the presence of acylating agents. In the enzyme the O-O bond cleavage is promoted by nearby amino acid residues [9]. In both these examples the formation of the high valent metal centre

was driven by reducing the metal centre analogous to Figure 2. Synthetic analogues of course have no substrate specificity, hence the need to use P450 enzymes however these studies do tend to indicate that electrochemically driving the cycle shown in Figure 2 should be possible without significant short circuiting. Also in cytochrome P450s the substrate is typically bound in a pocket 2-3 Å from the heme. Thus the rates of oxygen insertion by P450s are significantly faster than in porphyrin analogues. For example in cytochrome $P450_{cam}$ the rate constant of oxygen insertion is of the order of 10^{10} s^{-1} [10].

The effect of dioxygen

When P450 is immobilised on an electrode the voltammetry in aerated buffer shows diffusional tailing and no reverse reaction (Figure 3).

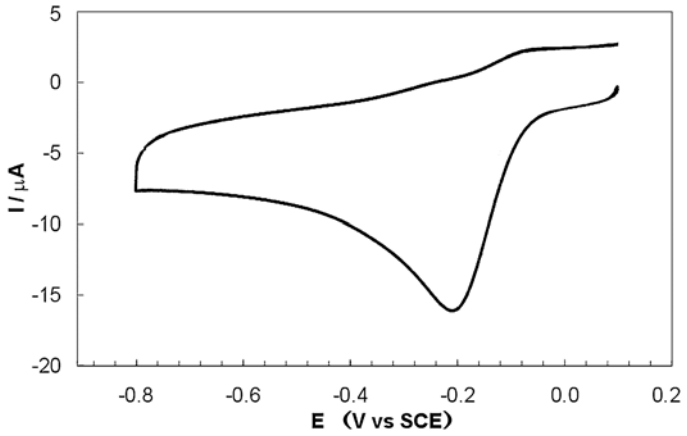

Figure 3. CV of $P450_{BM3}$ immobolised on a graphite electrode in aerated 50mM phosphate buffer pH7.4

This response is due to the P450 heme catalysed mass transport limited reduction of dioxygen. The mass transport limitation causes the diffusional tailing in the measured voltammetric current, a phenomena in voltammetric experiments that has been studied and understood for decades [11].

As a thought experiment imagine that you've recorded the P450 catalysed dioxygen reduction voltammogram and then added some substrate to the electrochemical cell. Should we expect to observe a change in the electrochemical response? Well

possibly – the binding of substrate should cause a small but detectable shift in the redox potential of the Fe^{3+} reduction and this should shift the dioxygen reduction wave. But what of the kinetics? Prior to adding the substrate to the solution the electrochemical response was that of a "classic" mass transport limited reaction. If a reaction is mass transport limited their are only two ways and increase in the response can be observed: either the rate of mass transport must increase or the bulk concentration of the analyte must increase. Cyclic voltammetric experiments are carried out in quiescent solutions so as to eliminate convection and make diffusion the sole means of mass transport. It follows that the only way the amount of dioxygen reaching the electrode can be increased is to increase the bulk concentration dioxygen.

Adding substrate to the solution can either increase the rate at which dioxygen is turned over by the enzyme or reduce that rate. If the presence of the substrate increases the rate of turnover of dioxygen then we still observe a voltammogram for the mass transport limited reduction of dioxygen. If, in the presence of the substrate the enzyme kinetics becomes rate limiting producing a decrease in the rate of dioxygen turnover then we may observe a reduction of the dioxygen catalysis.

Due to the hydrophobic nature of P450 substrates, stock solutions of substrates are normally dissolved in organic solvents. The solubility of dioxygen in many organic solvents is much greater than in aqueous solutions [12]. Therefore adding an aliquot of a substate stock solution to an electrochemical cell is likely to produce a small transient increase in the dioxygen concentration in the cell solution. Thus a similarly small increase in the mass transport limited current is likely. Unfortunately no attempts seem to have ever been made to measure and control the dioxygen concentration during the course of adding substrate to the cell. Nor have any studies reported the results of control experiments in which aliquots of the solvent used to dissolve the substrate have been added to the cell.

In the absence of any explanation as to how it would be possible for the addition of substrate to an electrochemical cell to increase the voltammetric current (which is due to the mass transport limited reduction of dioxygen), and in the absence of any

control experiments in which the dioxygen concentration is monitored, the principle of occams razor suggests that the small increases in current that have occasionally been observed must be due to small increases in dioxygen concentration, probably from dissolved dioxygen in the solvent used to dissolve the substrate.

The P450 non-turnover response

The initial experiment in an electrochemical study of P450 employing direct electrochemical methods involves the immobilisation of the enzyme on an electrode surface. Generally methods such as physical adsoprtion directly onto an edge plane graphite surface, immobilisation within a surfactant film, attachment via a histidine tag, cysteine surface modification of the enzyme followed by chemisorption on gold have been " successfully" employed.

We have no way of knowing, based on reported electrochemical experiments, what conformational form P450 takes on an electrode surface. Spectroscopic methods such as various surface reflectance methods may yield information however these experiments are likely to be hampered by both the nature and the morphology of the (mostly) graphite surfaces used as electrodes in P450 direct electrochemistry and the intrisicly small effective path length being employed when only a monolayer of P450 exist on electrodes.

Spectroscopic characterisation of the enzyme in a film on, say a glass slide, does not tell us what happens to the enzyme when it comes into contact with the electrode surface. Such experiments do however provide valuable information about the stability of the enzyme within various films and coatings.

Having observed an electrochemical response, characteristic of an adsorbed species, following the immobilisation of P450 on an electrode where to next?

- Since the heme in P450s is not covalently bound one needs to rule out that the response is due to free heme that has become dislodged from the enzyme during the adsorption process. The next step should therefore be to compare the voltammograms of P450, of hemin, and of P450 plus hemin modified electrodes.

Even if the heme has not been dislodged from the enzyme how do we know that the enzyme has retained its catalytic conformation following adsorption?

- The voltammetry of P450 and P420 should be to compared under identical conditions. Figure 4 shows that voltammograms of $P450_{cam}$ and $P420_{cam}$ have indistinguishable redox potentials. Given the lack of robustness of P450 how can one conclude that the response recorded after placing P450 on an electrode is not actually due to P420 that has formed from the P450 during the adsorption process and/or under the influence of the electric fields present at the electrode solution interface?

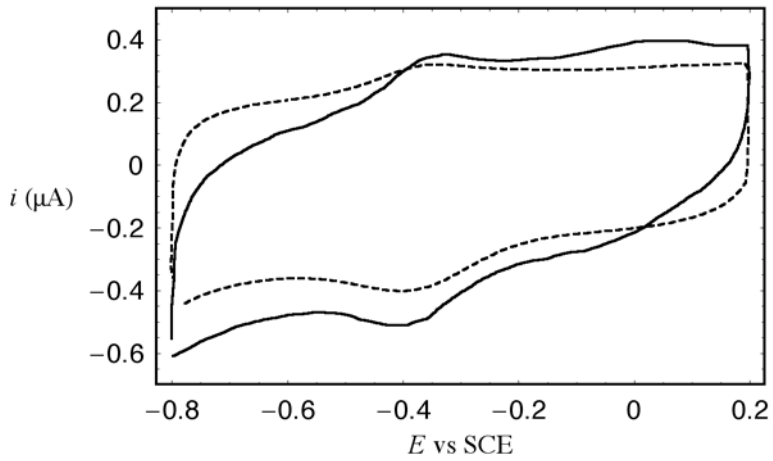

Figure 4: Cyclic voltammograms of $P450_{cam}$ (---) and $P420_{cam}$ (—). Each enzyme was immobilised on a poly(lysine) modified edge plane graphite electrode. Voltammograms were recorded in 0.1 M potassium phosphate buffer pH 7.4; sweep rate was 50 mV s^{-1}.

If it is not possible to distinguish between P450, P420 and/or free hemin from non-turnover electrochemical experiments the existence of enzymatically active enzyme at the electrode surface can only be established by monitoring the products of the (presumed) electrochemically driven enzyme catalysis.

Direct electrochemically driven catalysis

As discussed above we know of no mechanism described in the literature that could possibly explain an increase in the voltammetric response when substrate is added to an aerated buffer solution. Nevertheless if electrochemically driven catalysis is

occuring then the product of the enzyme reaction could be detected in the electrochemical cell solution.

It has become common for catalysis of non-natural substrates to be reported. The problem with using non-natural substrates is that they do not result in experiments that potentially produce unequivicol answers to our question, “is the P450 functioning as an active enzyme on or at the electrode?” We must remind ourselves that in using enzymes immobilised on an electrode we are presumably endeavouring to electrochemically drive a stereo or regio specific catalytic reaction. Thus we are not endeavoruing to demonstrate catalysis *per se* but very specific catalysis of the type that only enzymes generally perform.

Ultimately non-natural substrates may be of more interest, particularly when considering the use of P450s for biosensors and biocatalysts but surely a first step would be to demonstrate the enzyme functionality with the natural substrate because many non-specific catalytic reactions can also be performed by hemin and heme containing proteins such as myoglobin. So if non-natural substrates are being used we should be asking “why is an apparent electrochemically driven catalytic turnover significant?” For example why is it significant that P450 immobilised on an electrode will dechlorinate a chlorinated hydrocarbon if myglobin will also do this? Couldn’t the same result have been achieved by a partially denatured P450 or any protein with a solvent accessible five co-ordinate heme?

The only possible reason for using P450 in these reactions would be if the P450 functioned enzymatically to produce products that were stereo or regio specifically different to products that were derived from non-specific catalysis using, for example, free hemin or myoglobin or even P420. And how would P420 perform under identical conditions? Would it also catalyse these reactions? So unless it is established that the products of the catalysis of non-natural substrates have some stereo or regio significance relative to products formed from other heme protein catalysts the reporting of successful catalysis does not imply that the success was due to immoblised “conformationally native” P450. Control experiments need to be performed.

Finally it is possible that despite performing all the control experiments described above product turnovers may be achieved indirectly via the "peroxide shunt" mechanism. In this mechanism hydrogen peroxide oxidises Fe^{3+} directly to the high valent oxyferryl form. Therefore in an electrochemical experiment it maybe possible, indeed probable, that hydrogen peroxide formed at the electrode surface via dioxygen reduction would react chemically to drive some turnovers. The result of catalysis via this mechanism would be a detectable amount of product in the cell solution. The drawback of the peroxide shunt mechanism is that the presence of peroxide eventually "poisons" the enzyme so that the usefulness is limited. The best way to try and eliminate the peroxide shunt mechanism from being a major contributor to catalysis may be to add the peroxide consuming enzyme catalase to the electrode coating by coadsorbing a catalase – P450 mixture.

Summary of control experiments.

Any reports of the electrochemistry of P450 immobilised on an electrode in the presence or absence of substrate, dioxygen, carbon monoxide and so on should be accompanied by control experiments in which identical studies have been carried out, at the very least with P420, but ideally with hemin and also a heme protein such as myoglobin. When determining the effect of adding substrate a control experiment is required in which an aliquot of the solvent used to dissolve the substrate is also added to the cell. If at all possible the dissolved dioxygen concentration should be monitored.

A four electrode cell should be used comprising two working electrodes, one with immobilised P450 and the other with immobilised P420, a reference electrode and a counter electrode. Voltammograms can then be measured under identical conditions by switching between the two working electrodes.

This experiment should then be repeated using myoglobin instead of P420.

In another experiment catalase should be co-immobilised with P450 and P420 and a small amount should be added to the cell solution. The aim is to reduce or eliminate hydrogen peroxide.

In the absence of these control experiments it is simply not possible to assert that what was observed was the direct electrochemistry of native cytochrome P450.

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